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During the second year of our Army support, we have made substantial progress toward isolating tumor-reactive lymphocytes, and toward developing a model system by which various notions regarding immunotherapy can be tested. All of this most recent work depended completely upon the development of a SCID mouse animal model which sustains the growth of many patients' primary breast tumors, and the expansion of this model to include a larger number of patients (which was achieved during the first year of support). A publication which describes in detail this model and our other relevant findings has now appeared in print. During this past year, we correlated the growth rate of tumors in the SCID mouse with nodal status, estrogen receptor status, and prior exposure to chemotherapy. We also began collecting not only peripheral blood from breast cancer patients, but also draining lymph nodes and leukapheresis specimens for the isolation of tumor specific T cells. In partial fulfillment of the goals of Aims 1 and 2, we have now developed several T cell lines and clones which are stimulated by autologous tumor, and can lyse autologous tumor cells. These T cells are being added to SCID mice bearing tumors to determine their killing efficiency *in vivo*. We have begun to characterize the human antibodies found in the SCID mouse sera using western blots of autologous breast tumor, and other solid tumors in an attempt to identify new antigens. Finally, we observed that a long term, low temperature hyperthermia treatment (fever-range exposure) can induce a substantial amount of heat shock proteins in the patients' breast tumor. This treatment also appears to cause a dramatic increase in the killing of tumors by host NK cells. We are agressively pursuing this unexpected finding as part of the goals related the use of heat shock proteins in Aim 3.

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Elizabeth Ryan 10/29/96

PI - Signature Date

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INTRODUCTION

DEVELOPMENT OF THE MODEL

Pre-clinical research of the treatment of human breast cancer has been hampered by the lack of an appropriate experimental animal model in which the growth of primary breast carcinoma biopsies can be studied. Compounding this situation, *it is very difficult to obtain enough primary breast tumor cells for most kinds of studies, including antigen identification or RNA and DNA isolation.*

Most studies on human breast cancer rely on a few established immortal human breast cancer cell lines. The few cell lines which have been established in long-term culture, and which do grow in immunodeficient mice, most have been obtained from malignant pleural effusions. Although the majority of studies on human breast cancer are conducted on these cell lines, they may bear little resemblance to earlier malignancies found in primary breast carcinoma itself. An animal model that sustains the growth of patient breast tumors could be used not only to study the cellular and molecular properties of various breast carcinoma specimens but also could be used to evaluate the effect of various immuno- or chemo-therapeutic agents or of various factors (such as diet) on breast tumor growth. Moreover, there is a great need for earlier detection markers and better prognostic indicators in the clinical evaluation of human breast carcinoma. Knowledge of biological differences among various primary breast cancers would help to identify such factors and help in the choice of the best treatment modality.

Despite a longstanding effort, human breast carcinomas have proven uniquely resistant to growth in a variety of animal model systems including the anterior chamber of the eye of guinea pigs, lethally irradiated or thymectomized mice and nude and SCID mice (reviewed in the reprint attached to this proposal; Sakakibara *et al.*, 1996) even when the animals are supplemented with estrogen. Apparently, human primary breast carcinoma cells require a vary special micro environment for growth that has not previously been replicated in animal models or *in vitro*.

At the time our original Army grant was submitted, we had been testing a variety of protocols attempting to improve the chance of growing human primary breast carcinomas by using SCID mice. Our initial and extensive attempts to engraft human breast tumors in SCID mice by inoculating tumor biopsy tissue or dispersed tumor cells subcutaneously or intraperitoneally were largely unsuccessful, confirming an earlier study by Phillips. However, a successful technique was eventually developed in which surgical specimens of human breast carcinoma were embedded in the gonadal fat pad of SCID mice. Based on our preliminary data (using seven patient's tumors), we submitted the original Army grant.

In this grant, ***our major goal was to use this model system to develop a model for the study of patients' immunity to their own breast cancer.*** During the first year of support, we expanded our data base to include well over 40 different patients. Importantly, we also began to observe the onset of metastasis in the model. For many of our fast growing tumors, we observed metastatic spread to the liver, lung, diaphragm and abdomen. We are currently evaluating mice for bone involvement, since bone metastasis is a very common event in patients with advanced breast cancer.

RATIONALE BEHIND THE APPROACH

Cancers of solid tumor origin continue to represent one of the major unsolved problems of modern medicine. For most of the major cancers (which include breast), there has been essentially no improvement in survival since 1930. For most of these cancers, it is possible to control the initial malignancy with surgical excision. Unfortunately, surgical methods are not as successful if the cancer recurs or spreads to other sites. This fact underscores the growing recognition that solid tumor cancer ***is a systemic disease, requiring systemic treatment***. Although there is already a systemic treatment (chemotherapy) applied to most cancer patients as a follow up to surgery, or as neoadjuvant therapy, it is evident that only a small fraction of patients are actually benefitting from this often debilitating treatment.

For at least a century, there has been strong appeal to the notion that an immune response to tumor might exist and develop akin to that observed in *infectious diseases*. However, implementation of immunotherapies designed to augment the body's own natural defenses in the treatment of human breast carcinoma and other solid tumors has been disappointing. The eventual strategy that is to be developed for cancer immunotherapy will apparently require a far more profound understanding of immune mechanisms and its regulation.

Studies of a variety of animal and human tumor models indicate that the immune response is capable of controlling tumors, particularly in animals that do not have a progressively growing tumor. However, tumors often continue to grow and spread despite the presence of large numbers of lymphocytes (including T, B and NK cells), plasma cells and macrophages in and around the tumor. While there are several possibilities to explain this situation, *the escape from immune recognition by human tumors represents one of the most intriguing areas of modern biology. Indeed, in virtually every tumor in which it has been carefully examined, there has been evidence of both immunologic responsiveness and escape from this response in established tumors*. Loss of individual MHC class I alleles, loss of cell surface molecules important for susceptibility to apoptotic death induction (Fas), loss of B2 microglobulin expression and peptide transporters required for maturation of the Class I molecule and presentation of antigenic moieties, and production of immunosuppressive cytokines have been identified in human tumors including epithelial neoplasms such as breast carcinoma, cervical carcinoma, and colon carcinoma (see review in 1). Even more disturbing is very recent data showing that the lymphocytes and other immune elements found within tumors are actually defective in their function.

More than a decade ago, investigators developed methods for the *in vitro* growth of tumor reactive cytotoxic T cell clones (1-4) , and the use of these clones in the treatment of tumor bearing animals (5) . Indeed, adoptive transfer of *in vitro* propagated cytolytic and helper T-cell clones has been shown to be effective in promoting tumor destruction in a variety of animal models (6) and in human clinical studies (7). The molecular characterization of a variety of T cell growth factors, most notably IL-2 (8) and their use in clinical trials has been of further use in promoting adoptive immunotherapy approaches including the use of lymphokine activated killer (LAK) cells and tumor infiltrating lymphocytes (TIL) in oncology settings (1, 9). While positive results have been seen with such adoptive immunotherapy approaches (most notably in patients with melanoma and in renal cell carcinoma (10)), widespread use of these techniques in common oncology practice has unfortunately been limited due to the large amount of technical sophistication required to grow sufficient numbers of tumor reactive cells ex-vivo as well as the necessity of administering toxic amounts of T cell reactive cytokines to patients in order to promote viability and functional reactivity of adoptively transferred

tumor reactive T cells. More recently, through the use of gene therapy approaches, investigators have attempted to simplify adoptive immunotherapy protocols by transfecting cytokine cDNA(s) in expressible form (predominantly via retroviral vectors) directly into tumor or lymphoid cells for re-infusion into patients (11, 12). However, several additional years of investigation will be required in order to delineate the safety and efficacy of these and related approaches.

One important spin-off of the above mentioned research has been the availability of reasonable numbers of tumor-reactive cytolytic T cells for other aspects of immunological and molecular characterization. Elucidation of the means by which CTL recognize and bind antigen (through the antigen binding cleft of the Class I MHC molecule) has *led to the use of tumor-reactive CTL as a means of defining tumor antigens*. By stripping peptides from Class I MHC expressed on tumor cells, investigators have identified a number of potential tumor antigens which might hold promise as putative cancer vaccines (13). Indeed, even peptides from subdominant regions of self antigens which are over expressed on tumor cells (e.g. peptides from HER-2/neu) have been shown to stimulate CTL and helper cell responses from cancer patients and in animal models of tumor immunity (14). *Despite these advances, the process of identifying tumor antigens which might serve as potential vaccines remains extremely cumbersome given the reliance on large numbers of tumor-reactive CTLs, and sophisticated biochemical approaches for stripping and identifying class 1 MHC-bound peptides*. The translation of this approach is additionally problematic because it requires that tumors be obtained in large quantities that are capable of being made into single cells. This problem is quite significant, *particularly* in the United States, where surgically excised breast tumors are generally small, and by the time appropriate amounts of the excised tumor have been selected for clinical pathology, available material for preparing cells for *in vitro* culture is usually insufficient and there is little or no chance to have enough material to repeat the experiment.

In our original Army proposal, we proposed to use a newly identified SCID mouse model to help determine effective immunotherapy strategies for patients, since we had found a way to sustain a larger number of patients' breast tumors in SCID mice.

Our earlier experience supported the idea that this SCID mouse model of breast carcinoma could be used to 1) study the patients' own immune response to her tumor and 2) identify strategies to enhance this response in a pre-clinical setting. This is dependent on the inclusion of the patients' own immune cells which have infiltrated the tumor being co-engrafted into the SCID mouse with the tumor specimen.

Brief summary of first year progress: As detailed in the October 1995 report, substantial progress was achieved towards the goals of the grant in the first year. Specifically, these include the successful expansion of a SCID mouse/ human breast cancer tumor bank. A large number of patients' primary and metastatic tumors were implanted and have been sustained through several passages. Concurrently, this has also been accomplished for several other types of solid tumors, and this is an invaluable resource for the experiments outlined below. We carried out histological and phenotypic analysis of the tumors, including identification of the immune cells (lymphocytes) which co-engrafted with the tumors.

BODY

Technical Objectives of the Original Grant:

1) To characterize the SCID mouse as a model for the growth of human breast cancer and autologous immunocompetent cells and correlate tumor growth parameters with patient's prognosis and expression of predictive markers of tumor malignancy.

2) To demonstrate and monitor specific anti-tumor reactivity of the human immunocompetent cells that are co-engrafted with autologous human breast tissue, and correlate these findings to the patient's prognosis and expression of predictive markers of malignancy.

3) To enhance the anti-tumor immunity of human immunocompetent cells in SCID mice by 1) *in vivo* or *in vitro* transfection of human breast tumor cells with genes encoding immunostimulatory proteins and 2) *in vitro* priming of immunocompetent cells with tumor antigens.

In the second year of the grant, we made progress in each aim, and toward each goal in the Statement of Work. During this past year, we correlated the growth rate of tumors in the SCID mouse with nodal status, estrogen receptor status, and prior exposure to chemotherapy. We also began collecting not only peripheral blood from breast cancer patients, but also draining lymph nodes and leukapheresis specimens for the isolation of tumor specific T cells. In partial fulfillment of the goals of Aims 1 and 2, we have now developed several T cell lines and clones which are stimulated by autologous tumor, and can lyse autologous tumor cells. These T cells are being added to SCID mice bearing tumors to determine their killing efficiency *in vivo*. We have begun to characterize the human antibodies found in the SCID mouse sera using western blots of autologous breast tumor, and other solid tumors in an attempt to identify new antigens. Finally, we observed that a long term, low temperature hyperthermia treatment (fever-range exposure) can induce a substantial amount of heat shock proteins in the patients' breast tumor. This treatment also appears to cause a dramatic increase in the killing of tumors by host NK cells. We are aggressively pursuing this unexpected finding as part of the goals related the use of heat shock proteins in Aim 3.

Experimental Methods:

Nearly all of the methods for characterizing tumor growth for Aim 1 are detailed in the attached reprint. For the work conducted for Aim 2, we are using isolation of T cells derived from draining lymph nodes and from leukapheresis. We have IRB protocols to obtain these materials for these studies which are being done in collaboration with surgical and medical oncologists here at RPCI. To expand and stimulate these T cells for the identification of CD8 positive T cells, we are following *in vitro* culture methods that are detailed in several published works from Thierry Boon's group and that of others (See review of these studies for melanoma studies in ref. 15).

For the hyperthermia work being conducted as part of Aim 3, we have attached an Abstract which describes the method of study and the core temperatures obtained.

Results and Discussion:

Progress toward accomplishing Aim 1:

To determine if there were any factors useful in predicting whether a given patient's tumor would grow quickly, slowly, or not at all in SCID mice, we evaluated various available clinical data, including node status, ER expression, and whether or not the patients had previous treatment (e.g. chemotherapy) prior to surgical removal of the tumor.

We observed that of the 36 primary, untreated cancers transplanted (see Table 1 in the attached reprint) 5, (14%) grew rapidly, 21 (58%) grew slowly, and 10 (29%) failed to grow. In comparison, of the six primary tumors from patients who had received chemotherapy previously, four (67%) were rapid growers, two (33%) were slow growers, and non failed to grow ($P = 0.011$, Chi square). Being ER negative predicted rapid growth (8 of 19 (42%) while only 3 of 25 (12%) ER-positive tumors did so ($P = 0.035$; Fisher exact test). The presence of axillary nodal metastasis was not predictive (5 of 28 node-positive tumors (18%) grew fast and 3 of 12 node-negative tumors (25%) did so ($P = 0.677$, Fisher exact test).

Progress toward accomplishing Aim 2:

Identification of human lymphocytes within SCID mice engrafted with breast tumors: We have observed the presence of large numbers of human lymphocytes and plasma cells in the SCID mice engrafted with human breast tumors. Often, these cells can be found distributed around the periphery of tumor cell clusters in animals in which the tumor has failed to thrive (see Fig. 1). The majority of these lymphocytes are of human origin as demonstrated by immunohistochemical staining (data not shown). Further characterization of these cells is now being carried out by Dr. Yan Xu in our laboratory.

Isolation and characterization of T cells which are stimulated by, and can lyse, autologous tumor cells. During this past year, we isolated and characterized the autologous T lymphocytes that were collected from either draining lymph nodes or patients' blood obtained by leukapheresis. A table summarizing all of our breast tumors for which we have isolated T cells from the patient (or have developed T cell lines and clones) is attached to this report (Table 1).

Substantial progress has been made in generating tumor specific T cell lines and clones. Figure 2 shows that T cells generated from the draining lymph node of a breast tumor secrete IFN-γ in response to autologous tumor cells.

Characterization of human antibodies found in the serum of SCID mice implanted with human tumors. We have observed that human antibodies are present in the serum of mice implanted with human breast tumors (based on ELISA analysis). Because the source of the B cells and plasma cells that are producing this antibody must have been the infiltrating TIL cells that were co-implanted with the tumor, we believe that these sera could be an important source of tumor reactive antibodies which may indicate important new antigens. To study these antigens, we have begun to conduct Western blot analysis of the patients' breast tumors and various other tumors and control tissues. As seen in Fig. 3, pooled sera from human breast implanted mice recognizes antigens of both breast and prostate tumors. Several bands have been identified using this technique, and we have established a

collaboration with Dr. Ken Grabstein of Corixa Corporation to conduct expression cloning of these tumors to help identify these potential new antigens.

Progress toward accomplishing Aim 3:

In Aim 3, our goal is to develop effective strategies for increasing the immunogenicity of tumors.

As described in Burd *et al.* (Abstract attached), during the past 12 months we have unexpectedly learned that we can cause a substantial and rapid reduction in tumor volume by exposing the mice to a whole body hyperthermia in the fever range (i.e, at a maximum internal temperature of 39.5-40°C) for 6-8 hours. We have also learned that NK cells appear to be responsible for the heat-induced tumor killing. We have observed a massive induction of heat shock proteins in the tumor in response to this treatment. We are currently evaluating whether the increased NK cell lytic activity is due to their recognition of heat shock proteins at the tumor, or whether there is a natural induction of NK cell activity in response to fever-like conditions.

CONCLUSIONS

Our work continues to support the notion that the SCID mouse can be an effective model system for the study of various aspects of patients' immune response to breast cancer (including T lymphocyte responses, antibody production and the potential role of NK cells in tumor cell killing following whole body hyperthermia. Key to these studies has been the ability to sustain the growth of primary breast tumor cells (as a source of antigen) and the TIL (for antibody production). Most of our efforts during year two were spent isolating and characterizing T lymphocytes from patients from whom we already had obtained autologous tumor. Using this tumor material as a source of antigen, we have now begun to isolate and characterize several T cell lines and clones. These cells are now being evaluated for their tumor killing potential *in vivo* and *in vitro*.

As we move into the third year of funding, the differences we find among patients' lymphocyte responses and whether or not infiltrating lymphocytes were able to make anti-tumor reactive antibodies will be correlated to patients prognostic variables and other clinical parameters. During the past year (as presented above), we analyzed several clinical factors that may be predictive of whether a tumor grows quickly or not at all. We found that a high proportion of tumors that grow quickly are ER-negative and derived from patients who had received previous chemotherapy. Since previous reports on the growth of patients' breast tumors in nude and SCID mice have not included this type of additional clinical data, we cannot compare our results or determine if these factors may play a role in the enhanced growth potential observed in this study. It is important to note that even with chemotherapy or the absence of estrogen receptors, tumors would still often fail to grow if implanted subcutaneously, so there appears to be a growth advantage for even these tumors if implanted within the gonadal fat pad. These results suggest that future reports should include additional clinical data with regard to patient specimens that are used, since these factors may play a role in whether a tumor grows quickly, slowly, or not at all.

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APPENDICES

Figures 1 - 3

Fig. 1: Histology of human breast carcinoma growth in the SCID mouse

Fig. 2: Tumor antigen induces IFNγ secretion from lymph node derived CD8+ lines

Fig. 3: Western analysis using sera from SCID mice bearing patients' prostate and breast tumors

Table 1: Breast Tumors in scid Mice

Two Abstracts:

1. Effect of long duration, fever-range, whole body hyperthermia on patients' carcinmas and the innate immune response by Burd, B., Xu, Y., Caligiuri, M., Subjeck, J. and Repasky, E. Presented at the 11th Annual Meeting of The Society for Biological Therapy held at Georgetown University Conference Center, Washington, DC, October 23-27, 1996.
2. Engraftment of patients' solid tumors and autologous lymphocytes in severe combined immunodeficient mice. Xu, Y. and Repasky, E.A. Submitted for the Keystone Symposia: Cancer Immunology and the Immunotherapy of Cancer, Copper Mountain, CO, Feb. 1-7, 1997.

Reprint:

Sakakibara, T., Xu, Y., Bumpers, H.L., Chen, F-A., Bankert, R.B., Arredondo, M.A., Edge, S.B. and Repasky, E.A. Growth and metastatsis of surgical specimens of human breast carcinomas in SCID mice. The Cancer J. *from Scientific American* **2:** 291-300, 1996.

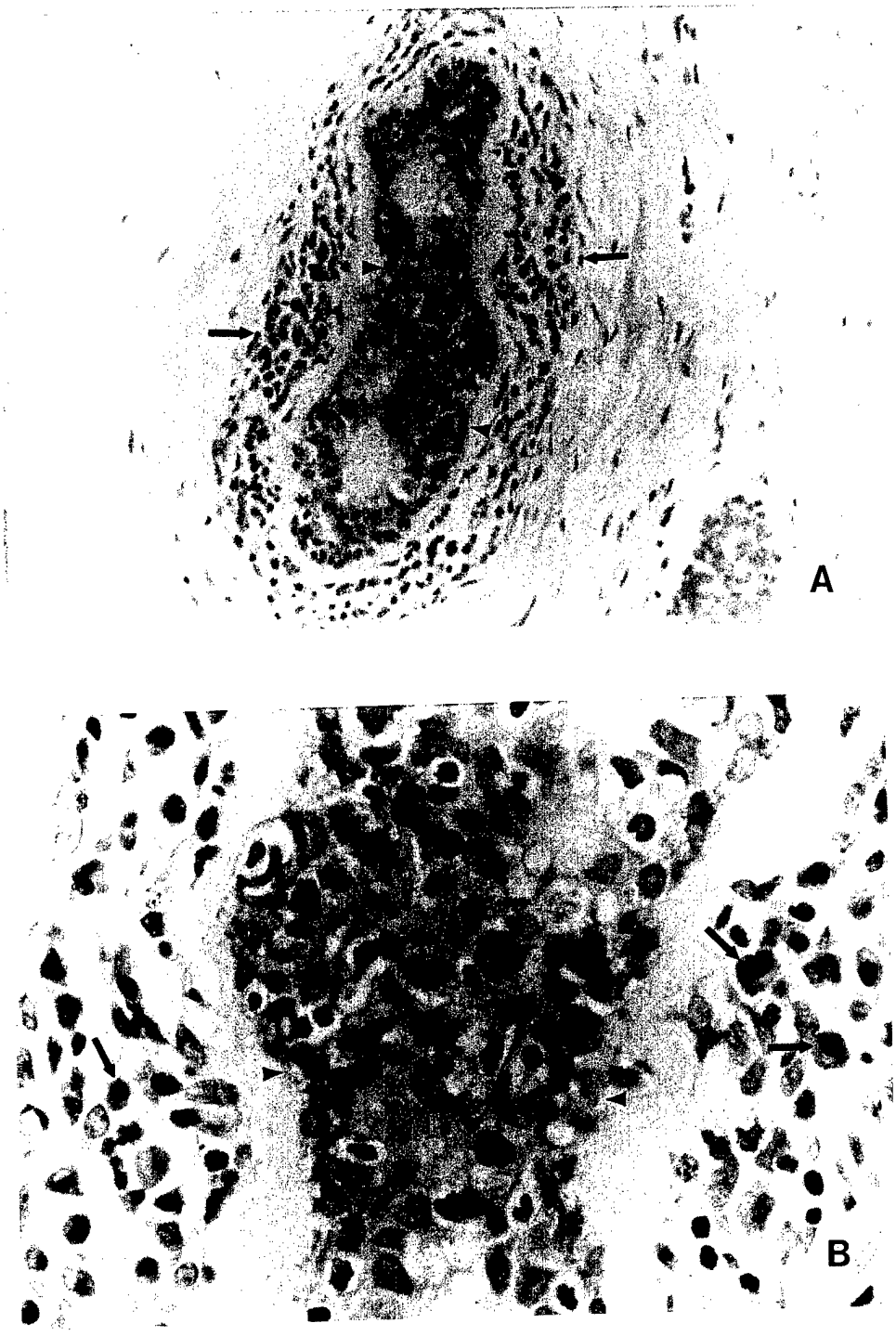


Figure 1: Histology of human breast carcinoma growth in the SCID mouse. **A.** Tumor cell cluster (arrow heads) surrounded by human lymphocytes (arrows) which had infiltrated the original tumor. **B.** At a higher magnification individual plasma cells (arrows) can be identified.

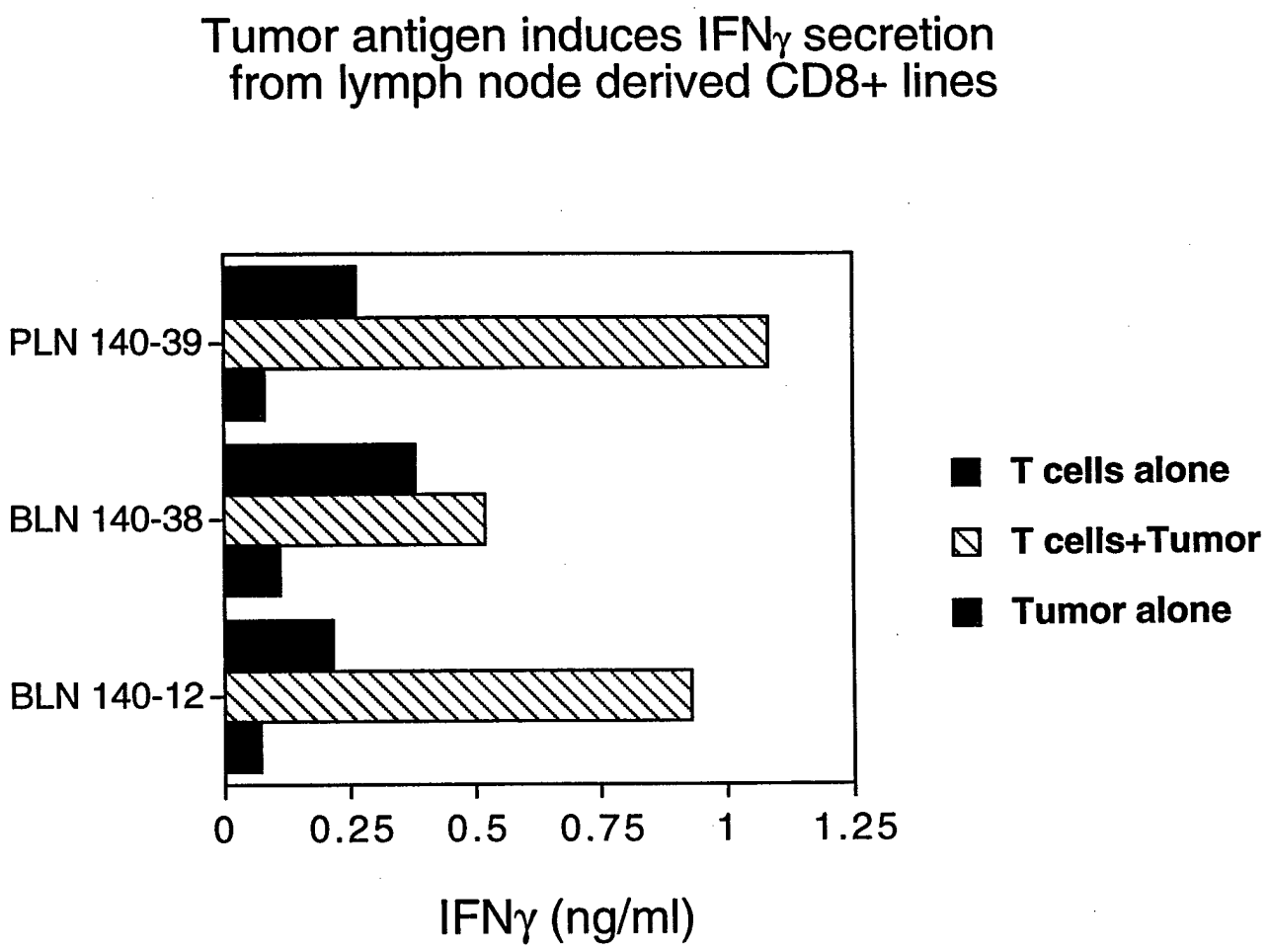
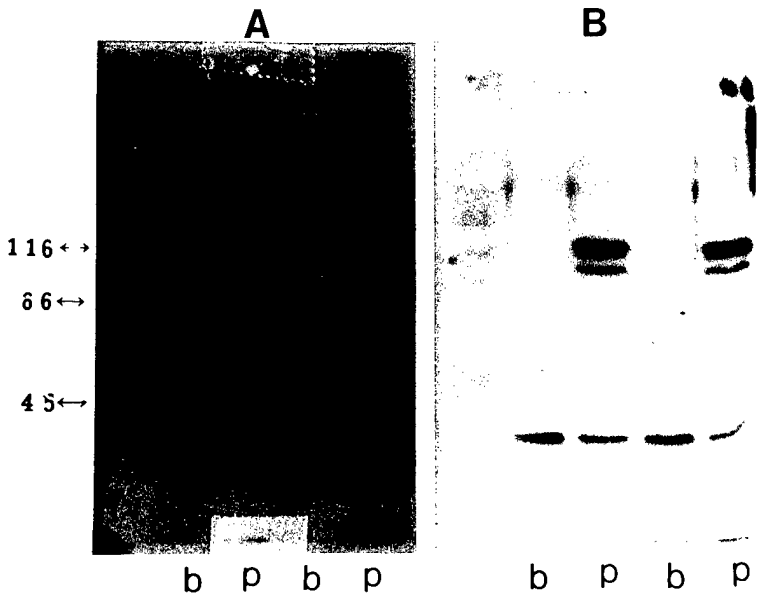


Figure 2. Lymph node cells were cultured in IL-2+IL-7 and restimulated with autologous tumor and APC. 140-38,39 were restimulated once prior to stimulation for assay. 140-12 was restimulated 5X.

FIGURE 3

Western analysis using sera from SCID mice bearing patients' prostate and breast tumors



Human Ig in sera of human tumor bearing mice recognized human tumor proteins. We took both human breast and prostate tumors from SCID mice and ran Western blots. On blots A and B, 1 and 3 are human breast tumor lanes (b) and 2 and 4 are human prostate tumor lanes (p). Blot A was reacted with pooled sera from mice bearing human breast tumors, and blot B was reacted with pooled sera from mice bearing human prostate tumors. HRP antibody against human Ig was used as the secondary antibody.

TABLE 1
breast tumors in scid mice

#	Received	PBMC	EBV	Serum	Lines	Clones	bulk	Patient #
BT 46-49	Tumor				yes	yes		(BK) 95M19307
BT 46-51	Tumor				yes			(DJ) 207668292
BT 46-66	Tumor	yes		yes	yes			
	Blood							
BT 46-78	Tumor	yes	yes	yes				
	LN							
	Blood							
BT 86-8	Tumor				no			(RC) 95529575
	LN							deceased
BT 86-14	Tumor				yes			(11/14/95) H30717
	LN							
BT 86-16	Tumor				yes			(DJ) H62991088 or
	LN							HR # H57-68-63
BT 86-28	none	yes						(BR) 8509
**BT 86-43*	Tumor	yes		yes	yes			(BR) 8600
	LN							
BT 86-58	Tumor				yes	yes	yes	96M5698
	LN							
**BT 86-62*	Tumor	yes		yes	yes			(BR) 8700
	LN							
BLN 86-80	LN				growing			(BK) 96M10554B
BT 140-13	Tumor				no			(SG)96M-14660
	LN							
**BT 140-21*	Tumor	yes		yes				(BR) 8867
	LN							
	none							(BR) 7443
	none							(BR) 7486
	none							(BR) 7628
	none							(BR)7722
	none							(BR) 7764
	none							(BR) 7914
	none							(BR) 7929
	none							(BR) 8038
	none							(BR) 8099
	none							(BR)8832
	none							(BR) 8848

Abstract submitted to the Eleventh Annual Meeting of The Society for Biological Therapy to be held in the Georgetown University Conference Center, Washington, DC, October 23-27, 1996

EFFECT OF LONG DURATION, FEVER-RANGE, WHOLE BODY HYPERTHERMIA ON PATIENTS' CARCINOMAS AND THE INNATE IMMUNE RESPONSE.

R Burd, Y.Xu M. Caligiuri, J. Subjeck and E. Repasky. (Roswell Park Cancer Institute, Buffalo, New York)

We have examined the effects of long duration, fever-range, whole body hyperthermia (WBH) treatment on patients' breast, colon and prostate carcinomas grown in SCID mice. Mice bearing breast tumors implanted into an abdominal fat pad were treated for 8h at 39.5-40^0 C (rectal temperature) in a gravity convection oven. Breast tumors treated with WBH had a 40% ($p<.05$) reduction in tumor mass 8d after treatment. We also examined patients' breast, prostate and colon carcinomas implanted subcutaneously. Treatment of each of these tumor bearing mice with WBH resulted in a rapid and substantial reduction in tumor volume coinciding with a significant increase in apoptosis of tumor cells. However, when anti-asialo GM-1 antibody (NK cell inhibitor) was injected prior to treatment, the tumor growth delay was abolished as well as the apoptosis. Human NK cells labeled with a Zynaxis fluorescent dye were injected into tumor bearing mice. Following WBH we observed a large increase in the number of NK cells within the tumors from treated mice, while few NK cells were observed in tumors from non-treated mice. Thus, fever-like WBH may be clinically useful, especially as an adjunct to immunotherapy by its ability to stimulate NK cell activity.

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Engraftment of Patients' Solid Tumors and Autologous Lymphocytes in Severe Combined Immunodeficient Mice

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To help develop improved models for the study of solid tumor immunity, we have analyzed the histology of over 250 human breast, prostate, lung and esophagus carcinomas implanted into SCID mice. We have observed that the growth potential and histology of the tumor xenografts and their extent of differentiation and vascularization can differ considerably, suggesting that solid tumor growth within SCID mice may recapitulate biological properties of the original tumors. We have also observed the presence of lymphoid cells among the tumor cells, and at various locations distant from the implantation site. Analysis indicates that these cells are mostly human T and B lymphocytes and plasma cells, and some murine cells. ELISA assay of the sera from engrafted mice shows that the majority of mice contain human immunoglobulin ranging from 5 to 400 μg/ml. Western blotting analysis shows that the human antibodies recognize human breast, prostate and lung tumor antigens. We have also studied the co-engraftment of tumors and autologous lymphocytes isolated from draining lymph nodes and peripheral blood of patients; our data indicate that tumor killing activity can occur in these mice following specific cytokine treatment and whole body hyperthermia. We believe that the SCID-human chimeric model will prove valuable for achieving a better understanding of immunological responses to malignant cells, and in the identification of tumor antigens. (Supported by US Army 17-94-J-4418)

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Growth and Metastasis of Surgical Specimens of Human Breast Carcinomas in SCID Mice

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■ **PURPOSE**

We have studied the growth and metastatic potential of surgical specimens of breast carcinomas engrafted into the large abdominal (gonadal) fat pad of severe combined immunodeficient (SCID) mice. We present results of this study, details of the implantation protocol and histologic characterization of several of the tumor xenografts.

■ **MATERIALS AND METHODS**

We evaluated the growth within SCID mice of 48 breast carcinoma specimens derived from 46 patients (45 primary breast cancers or local recurrences and 3 regional metastatic lymph nodes) obtained from resected tissues at this Institute over a 3-year period. The growth of each transplant was assessed by histologic examination of the xenografts at various times after implantation or upon passage into additional mice.

■ **RESULTS**

We observed that placement of human breast tumors within the gonadal fat pad could result in tumors that grew either rapidly, slowly, or not at all. Of 48 tumors studied, 12 (25%), including one of the three lymph node–derived tumors, grew rapidly enough within some or all of the implanted mice (i.e., the tumors reached a diameter of 2–3 cm within 2–6 months) to allow repeated passage. Metastatic spread to the SCID mouse lung, liver, and/or diaphragm and other sites was observed with the xenografts derived from 8 of these 12 rapidly growing tumors. Tumors in a second category often took from 6 months to over 1 year to only double or triple in size. This slow-growth group consisted of 25 patients' tumors (53%), including the remaining two metastatic lymph node–derived tumors. These xenografts would usually maintain a slow growth rate even upon later passage into new animals. A third category consisted of 11 patients' tumors (23%) that failed to grow at all (i.e., no evidence of tumor growth in any of the mice implanted), as discerned by histologic evaluation at various times after implantation.

Histologic examination of tumor xenografts and metastatic tumors revealed considerable variation in histopathology among the different patients' tumors.

■ **DISCUSSION**

Further examination of the heterogeneous properties of primary human breast carcinomas within SCID mice may provide a simple yet valuable new approach for the long-term study of human breast cancer biology. Importantly, use of the protocol described here can often permit the isolation of substantial quantities of human breast cancer cells for biochemical and molecular analyses. The ability to passage patients' breast tumors into large numbers of mice will permit the preclinical testing of new therapies for the treatment and prevention of this disease. *(Cancer J Sci Am 1996;2:291-300)*

Key words: Human breast carcinoma, SCID mice, metastatic breast cancer, gonadal fat pad, xenograft, estrogen.

Most recent experimental studies on human breast cancer rely on only a few established immortal human breast cancer cell lines; in particular, the endocrine-responsive MCF-7 line or the endocrine-independent MDA-MB-231 line. This dependence on only a few cell lines is due to the low success rate for establishing human breast carcinomas either as stable cell lines in vitro or directly as xenografts in immunodeficient mice.[1-5] Among the cell lines that have been established in long-term culture, and that grow in immunodeficient mice, most have been derived from the highly malignant cells found in pleural effusions. Because these cell lines were isolated from only a remarkably small subset of even the metastatic specimens, it is likely that they represent only the most aggressive subpopulation of breast cancer cells and bear little physio-

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logic resemblance to those found in primary or locally recurrent breast tumors.[1-3] Therefore, although the majority of cellular and molecular studies on human breast cancer are conducted on these long-term cultured, stable cell lines, the data derived are limited to the very latest stages of disease progression. Because many of these cells have been in continuous culture for many years, it is also likely that these cells may have undergone significant phenotypic changes in response to selection pressures of in vitro culture, rendering them even further distinct from patients' breast tumor cells.[2] The potential differences between breast tumor cell lines and primary tumors have recently been extended[6] to include mutations of tumor suppressor genes, such as the *CDKN2* gene that encodes the cell cycle regulatory cyclin-dependent kinase-4 inhibitor p16, because it was shown that the mutation is present in various cell lines but not in patients' tumors. Thus, despite the volume of work that has accumulated based upon the use of human breast cancer cell lines, it is clear that we cannot depend solely upon these cell lines for the best understanding of the cellular or molecular factors that regulate breast cancer progression in patients. Studies have described the isolation of cell lines from primary tumors, yet these systems are still few in number.[1,2,7,8]

In an alternative approach to the study of human breast cancer progression, investigators have examined the growth of patients' breast carcinomas directly implanted as xenografts into a variety of animal model systems, including the anterior chamber of the eye of guineapigs, lethally irradiated or thymectomized mice, and nude and severe combined immunodeficient (SCID) mice.[4,9-15] These approaches have also yielded a disappointingly low percentage of breast tumor growth compared to growth obtained with other major cancers of epithelial origin. In a comprehensive study that evaluated the growth potential of 433 human breast carcinoma specimens heterotransplanted into nude mice, Giovanella et al, reported that only 6.1% could be grown.[15] Two groups have analyzed the growth of human breast carcinoma implanted orthotopically (i.e., into the mammary fat pad) and both reported success.[16,17] However, in each study, a malignant specimen from only one patient was tested; thus, it is not possible to determine whether these results could be translated into a higher percentage of successful implants in a larger study. In the second of these studies, similar growth was obtained if pieces of the patient's tumor were placed subcutaneously or orthotopically; however, metastatic spread to the lung occurred in animals implanted orthotopically, an event that previously had not been observed.[17]

We have evaluated various protocols for improving the chances of obtaining growth and metastatic spread of human breast tumors by using SCID mice.[18,19] We report our findings of an evaluation of 48 human breast carcinomas implanted within the large, easily accessible abdominal (gonadal) fat pad (GFP) of SCID mice. The analysis reveals clear differences in the growth, histology, and metastatic potential among a variety of breast cancer patients' tumors, consistent with the clinical picture for this disease. The data obtained also indicate that use of this protocol can often result in the rapid and sustained growth, passage and metastatic spread of the implanted tumors. Factors that may affect rapid growth are discussed.

■ MATERIALS AND METHODS

Animals. The CB17-scid/scid mice used for this study were either purchased from Taconic Laboratories (Germantown, Penn) or were produced in the breeding colony at Roswell Park Cancer Institute. They were housed in microfilter cages (Lab Products, Maywood, NJ). All cages, water and mouse diet (Teklad Mills, Wienfield, NJ) were supplied after being autoclaved. The cages were maintained inside an air-conditioned and light-controlled (12 h/day) room. All surgical procedures were performed in a laminar flow hood. Female mice were used for this study. Except for certain experiments, each mouse was implanted with an estrogen pellet (1.7 mg 17-∃-estradiol, 2 mg cholesterol, Innovative Research of America) at the time of initial surgery and at the time of passage. Both virgin and retired breeder mice were used for this study, as no obvious differences were observed.

Tumor implantation. Fresh specimens of confirmed human breast malignancies (selected for this study only if the available specimen was large enough for implantation into at least two mice) were delivered to the laboratory shortly after their surgical excision. The tumors were sterilely minced with scissors (in RPMI 1640 containing 10% fetal bovine serum) to 1 to 2 mm sized pieces just prior to their implantation into subcutaneous sites and/or the gonadal fat pad of SCID mice. Mice were anesthetized via intraperitoneal injection of 0.35 to 0.45 mL of Avertin (2,2,2-Tribromoethanol, 12.5 mg/ mL) and placed on an operative metal cross. Typically, a 3-mm vertical incision was made on the right lower abdomen with scissors and the right portion of the gonadal fat pad was pulled up through the incision. One piece of tumor was implanted in the GFP by wrapping the GFP around the tumor and fixing it in place with 6-0 Dexon. The tumor+GFP was then replaced into the abdomen and the peritoneum was sutured. Skin clips were used to close the incision. At the time of surgery an estrogen pellet was placed subcutaneously on the back of the neck. After surgery, the mice were warmed until they awoke and then placed back into the cage.

Tumor growth and histology. Although tumor growth and volume could often be discerned by palpating the site of implantation, animals were killed at various time points and the tumor+fat pad was removed for histologic analysis or for passaging of the tumor

into other SCID mice. Because the number of mice implanted varied considerably, we did not have a uniform schedule of time points for histologic examination, but instead tried to equally space the time points for killing mice over at least 6 months to 1 year; longer time points were achieved (14 months). If mice died naturally during the course of study, the fat pads were removed as quickly as possible after the time of death and processed for histologic evaluation. For histology, excised tissues were fixed for 24 hours in 10% buffered formalin. The tissues were dehydrated for 18 hours by an automatic tissue processor (in an Autotechnicon) and then were embedded in paraffin blocks. Five micrometer paraffin tissue sections were obtained by using a microtome. The tissue sections were stained with hematoxylin and eosin.

In situ hybridization. To confirm human cells within the tumors, a biotinylated probe that recognizes human alpha-satellite DNA (p17H8) was used to react with tumor cells on frozen sections of tumor specimens.[20] Vecastain ABC kit (Vector Laboratories, Burlingame, Calif) was used for color development.

Analysis of keratin expression. Frozen sections of tumor specimens grown within the SCID mouse were stained for type II keratin using immunofluorescence labeled-AE-3 monoclonal antibody that is specific for an epitope found in type II keratin (kindly provided by Dr Bonnie Asch).

■ RESULTS

We evaluated a variety of protocols and sites within SCID and nude mice in the hope of increasing the growth potential of surgical specimens of human breast carcinomas. We were largely unsuccessful in either maintaining or growing any tumors (either disaggregated prior to injection, or as intact pieces) in subcutaneous sites in either nude or SCID mice, confirming the previous studies of others.[1,2,14,15] However, rare instances of tumor growth were observed if intact pieces of tumor were implanted within a mammary fat pad site in SCID mice. These preliminary efforts led us to conclude that implanting small cubes of intact tumor (as previously done by others[12]), rather than injecting disrupted cells, and finding a fat pad large enough to wrap completely around the intact segment of tumor were important factors for supporting more consistent growth. Therefore, pieces of patients' breast carcinomas were implanted within the large GFP found just under the peritoneum in the abdominal cavity. A total of 48 surgical specimens of human breast tumors were evaluated histologically at various times after implantation in this site (Table 1). Figure 1 shows the location of normal GFP and growth of one of the earliest human breast tumors implanted within this site. The histologic pattern of that particular tumor (7443; see Table 1) is also shown. However, we quickly noticed that there was extensive variability with regard to growth potential and growth rates among the various surgical specimens. In many cases, tumors exhibited little or no palpable growth for several months and then would begin to exhibit steady, yet remarkably slow, growth. The growth of some xenografts might have been missed altogether if external palpation were the only means used to assess growth; the abdominal location of these implanted xenografts makes palpation for their presence and growth more difficult until they have at least doubled or tripled in size. Often, growth could only be discerned via histologic analysis or by visual inspection of the isolated fat pad following dissection. In many cases, tumors would remain small for variable periods of time (1–5 months) and then suddenly exhibit fast growth; others would grow quickly from the first week after implantation. Moreover, we have successfully passaged most of the tumors with fast growth rates at least three times, several of the older tumors as many as 10 times, and currently continue to passage these tumors. It is also significant that within the group of mice implanted with pieces of the same surgical specimen there is varied success of tumor growth (i.e., for any given tumor, we observed that there were often one or more mice that did not sustain any growth, whereas mice implanted with a different piece of the same tumor may exhibit rapid growth).

We conducted a histologic evaluation of the engrafted tumors listed in Table 1. In addition to considerable variation in the rate of growth exhibited by various tumor xenografts, we also noted significant variation in the histology and vascularization of various tumors. The histologic patterns of the fast-growth tumors could vary considerably and, in addition to the tumor shown in Figure 1C, three additional patterns we observed are shown in Figure 2. Some tumors grow in rather diffuse patterns, with little or no overall organizational state (Figs. 1C and 2A); these tumors were often seen to be well vascularized, presumably from the blood vessels of the GFP. There appears to be no obvious encapsulation of these tumors, and the individual cancer cells appear to invade the interstitial spaces between adipocytes of the fat pad. However, distinctive differences in the histologic pattern of two other fast-growth tumors are shown in Figure 2. In one case (Fig. 2B), the tumor consistently grew as individual foci that became necrotic in the center, whereas in another case (Fig. 2C) the tumor grew as individual, elongated cords that did not become necrotic in the interior. Similarly, the histologic patterns seen when slow-growth tumors were analyzed varied considerably (Fig. 3B–D). Although it is our impression that obvious vascularization is not as frequent in tumors that grow slowly as in faster growing tumors, this conclusion has not been verified experimentally.

When many slow-growing and fast-growing tumors were analyzed at various times after implantation, certain common features became evident. Tumors that were excised 1 to 3 months after implantation often appeared similar to the tumor shown in Figure 3A; much of the interior of the original implant (whose borders can be seen adjacent to the adipocytes of the fat pad) was acellu-

Table 1. Pathologic Diagnosis and Tumor Growth in SCID Mice

Tumor #	Pathologic Diagnosis	Nodal Involvement	Estrogen Receptor ≥ 15 fmol/mg	Growth in SCID Mice[a]
7326P	ILC	-	+	0/2
7356[c]P	IDC	+	-	2/4
7418[c]P	IDC	-	-	1/2
7421P	Intraductal Ca (with microinvasion)	-	-	1/2
7443[b,c]R	IDC, recurrence after chemotherapy, RT	N.A.	-	4/4[d]
7486[b]P	IDC	-	-	5/5[d]
7526P	IDC	-	-	2/5
7528P,CT	IDC, prior chemotherapy for lung cancer	-	-	3/4
7543P	IDC	+	+	3/5
7553P	IDC	-	+	2/4
7556[c]P	Papillary cystic Ca	-	+	3/5[d]
7573P	IDC	-	+	0/5
7582P	ILC	+	+	4/5
7595P	IDC, matched with 7744	+	-	0/3
7608[c]P	IDC	+	-	2/2
7628R	IDC, recurrence after chemotherapy, RT	N.A.	-	5/5[d]
7655P	IDC	+	+	1/3
7661P	IDC	+	+	0/8
7679P	IDC	+	-	5/9
7717P,A	IDC, primary after chemotherapy	+	+	2/2[d]
7722[b]LN	IDC, regional nodal metastasis	N.A.	-	4/4[d]
7744P,A	IDC, primary after chemotherapy	+	-	6/10
7748P	ILC	+	+	4/4
7764[b,c]P,A	IDC, primary after chemotherapy	+	-	9/10[d]
7784P	ILC	+	+	2/2
7788P	IDC	+	+	5/5
7806LN, CT, RT	IDC, LN recurrence, after chemotherapy, RT	+	+	0/3
7821P	IDC	+	+	0/4
7841P	IDC	-	+	0/2
7842P	IDC	+	+	3/3
7857P	IDC	-	+	0/3
7869[c]P	IDC	+	+	6/8
7882P	IDC	+	+	2/2
7892P	IDC, mucinous	N.A.	+	0/3
7897P	IDC	+	+	1/3
7914[b]P,CT	IDC, prior opposite BRCA + chemotherapy	+	+	4/5[d]
7929[b]P	IDC	-	-	4/5[d]
7960P	IDC	+	-	0/2
7967P	IDC	+	+	2/9
8001P	IDC	+	+	1/2
8029[b,c]P	IDC	+	-	2/5[d]
8038P	IDC	N.A.	-	4/5[d]
8070P	IDC, atypical medullary	+	-	1/2
8099[b]P,A	IDC, primary after chemotherapy	+	-	5/5[d]
8180P	IDC	+	+	3/5
8194P	IDC, matched with 8419	+	+	0/2
8200LN,A	IDC	+	N.A.	3/5
8419LA	IDC	+	+	1/3

[a] Numbers refer to the animals in which growth by histologic evaluation was obtained in the first passage out of the total number implanted with pieces of the original surgical biopsy material.
[b] Showed evidence of metastasis to other organs in later passages.
[c] Tumors that expressed Type II keratin and were demonstrated to be human by in situ hybridization using a human DNA specific probe.
[d] Showed evidence of exceptionally fast growth, and were passaged multiple times.
Abbreviations: P, primary (no prior treatment); A, neoadjuvant chemotherapy (chemotherapy prior to tumor removal); LN, lymph noide; R, recurrence; N.A., not available because not done or done elsewhere prior to this study; RT, radiation therapy; CT, previous chemotherapy; ILC, invasive lobular cancer; IDC, invasive ductal cancer.

lar, and consisted of nothing but connective tissue (collagen), while most of the tumor cells were found on the periphery of the implant, adjacent to the fat pad cells. Whether the cells in the interior had died, or whether they migrated to the periphery in response to other stimuli is not known. In most of the slow-growing and fast-growing tumors that were evaluated, an eventual asymmetrical outgrowth into the GFP occurred (i.e., the tumor cells appeared to "spill out" from one region of the implant; Fig. 2B & C). The implant itself became nearly devoid of cells. The timing of this event is variable, and appears to be dictated by properties of each tumor.

Some tumors were found in a state similar to that shown in Figure 2A for as long as 6 months, whereas others appeared to progress to the stages depicted in Figure 3B & C within several months and then continued to grow at a very slow but inexorable rate. Often a SCID mouse bearing a particular tumor would die of other causes and the tumor would be removed and found in the stages shown in Figure 3B & C. We also found occasional examples where a relatively uniform outgrowth of new tumor from the collagen center, which is devoid of cells, had occurred (Fig. 3D). This small but healthy tumor was found after 14 months of remarkably slow growth in the SCID mouse. Therefore, it is apparent that if histologic analysis is not performed, a significant fraction of slow-growing tumors may go undetected. However, this caveat does not apply to the inability to detect subcutaneous growth in SCID or nude mice, where the ability to palpate tumors implanted subcutaneously is considerably more accurate.

The histologic pattern of each tumor was maintained through several passages in the SCID mouse (at this time over seven passages for the tumors shown in Fig. 2A & B and three passages for the tumor shown in Fig. 2C). Moreover, the pattern evidenced by the tumor shown in Figure 2B was found to be reproduced in metastatic sites within the SCID mouse (Fig. 4).

Occasionally, we found zones of apparently normal mammary epithelial cells growing as isolated duct-like structures within the GFP, or occurring between zones of tumor (Fig. 3E). These cells may have derived from normal cells still present in the biopsy or may represent early malignant cells that do not as yet reflect the phenotype of other invasive cells in the tumor. The histologic criterion indicating that there was no growth of tumor in a particular mouse is shown in Figure 3F. The original explant can be seen within the GFP, is composed of connective tissue only, often degraded in many sites, but with no evidence of tumor cells. In this study, 23% of the patients' tumors failed to grow in any of the implanted animals (Table 1).

Quite relevant to the clinical issues related to human breast cancer, many of the faster-growing tumors also exhibited eventual metastatic spread within the SCID mouse (Fig. 4 and Table 1); metastatic foci of tumor were observed by us in the liver, lung, diaphragm, adrenals, abdominal wall, and subcutis, in decreasing order of frequency. Bone marrow was not analyzed in this study, but its involvement is currently being investigated. With one exception (8029), these tumors did not exhibit metastases within the first passage in the SCID mouse. Metastatic spread was often not observed for many months or until the xenograft had undergone several passages (1–5 passages was the range in our analysis). Again with one exception (7722), the tumors that showed metastatic spread within the SCID mouse were derived from primary or recurrent chest wall breast tumors in patients with no clinical signs of distant metastasis. Although nodal involvement was seen in most

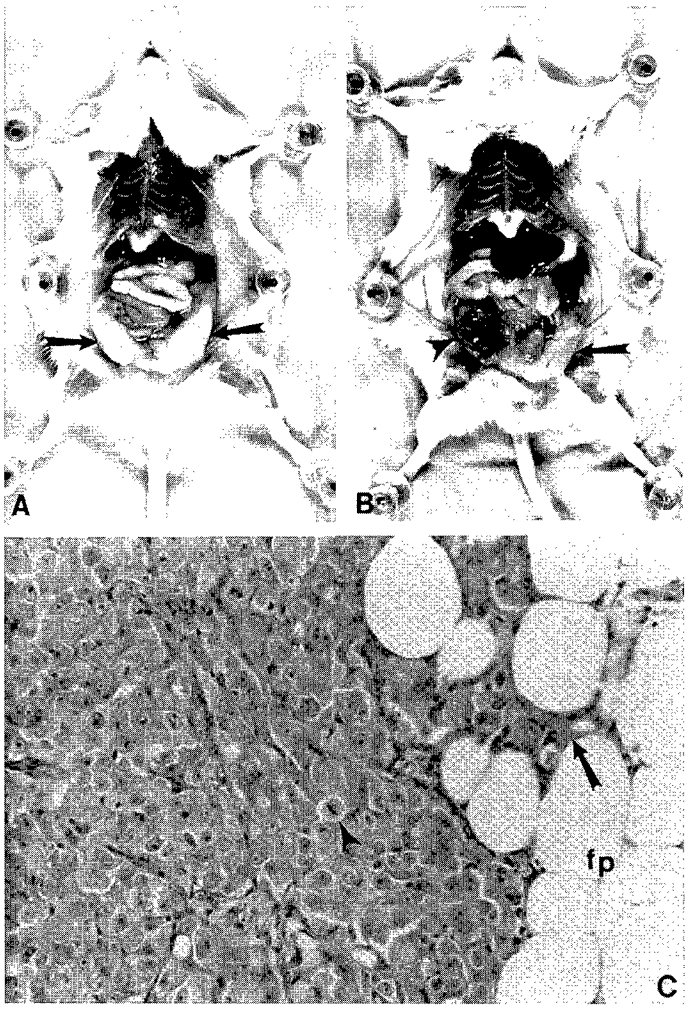

Figure 1 (A) Normal GFP of a 2-month-old SCID mouse showing its symmetrical positioning in the abdomen (arrows). (B) Arrowhead indicates a tumor seen 7 weeks after implantation of a surgical specimen (7486). (C) Histology of the tumor shown above indicates dense tumor growth, with nonencapsulated tumor cells invading the spaces between the surrounding adipocytes (arrow). Mitotic cells (arrowhead) are seen frequently. (Bar = 20 μm)

(but not all) fast-growing cases, this does not appear to be a critical factor for predicting growth rate or metastatic potential because nearly all of the patients whose tumors failed to grow whatsoever were also node positive (Table 1).

As with growth of patients' tumors in the fat pad, the metastatic tumors exhibited remarkable heterogeneity of growth patterns. They differed in which organ was most often involved (e.g., lung vs. liver) and also in the histology of the metastatic growth. Within the lung, a tumor may form one or two large foci of relatively undifferentiated tumor growth that are visible to the eye (Fig. 4A) or may grow as numerous, smaller micrometastases that are not visible upon gross inspection (Fig. 4B). Within the liver, we have observed foci of tumor that grow as nodules that are virtually identical to those seen in the tumor originally implanted into the GFP (Compare Fig. 4C with 2B, which are of the same tumor). Alternatively, liver metastasis can appear

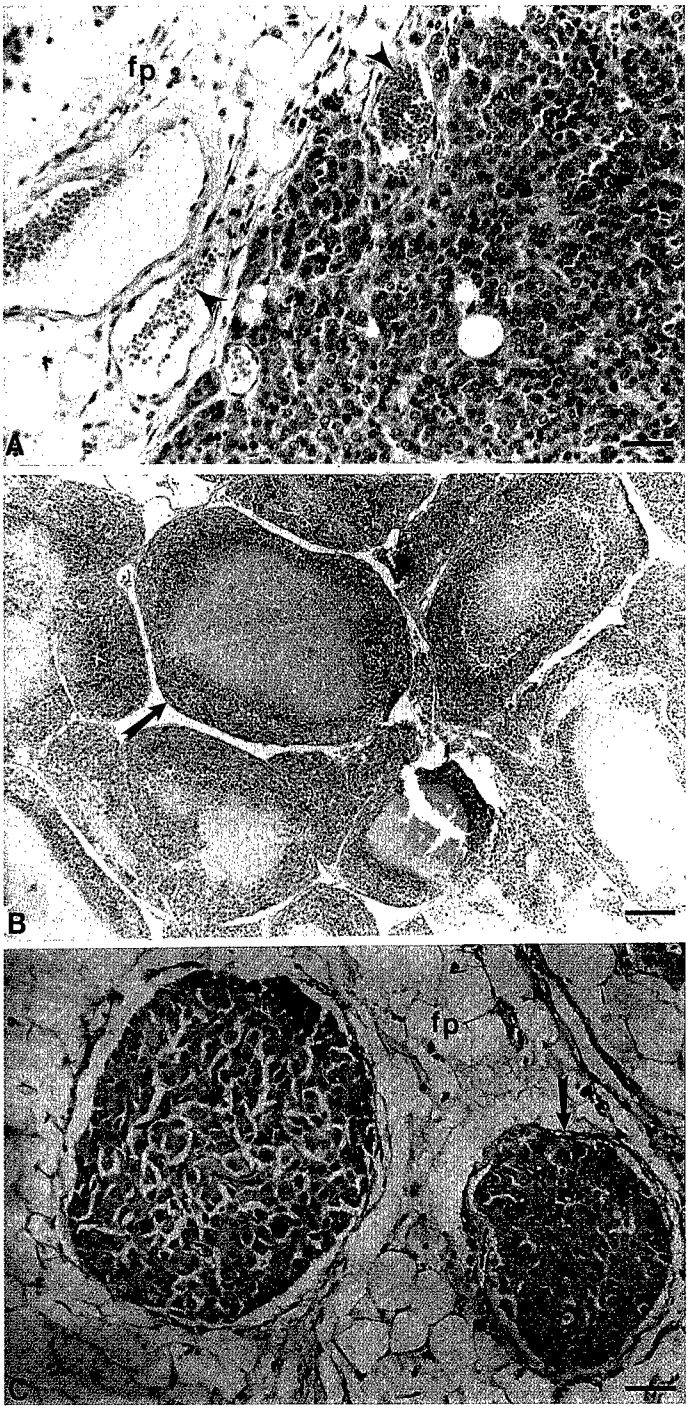

Figure 2 (A) This tumor (7743) exhibited a diffuse growth pattern similar to that seen in Fig. 1C, except that vascularization is more obvious (arrowheads). (B) In this example, (7722) the tumor grew in discrete foci that appeared necrotic in the center. Active mitotic growth is seen in the rim of tumor surrounding the necrotic tumor and an identical pattern was seen with this same tumor in metastatic sites (compare with Fig. 4C). (C) This tumor (7556) grew as elongated cords that intermeshed throughout the GFP. Unlike the tumor in Fig. A, the centers of these cords of cells did not usually appear necrotic. (Bar = 25 μm in A,C; 40 μm in B)

as a multiple large foci of tumor growth that appear sinuous, with connective tissue spaces evident, and without obvious necrosis.

In this study, we observed growth of both estrogen-receptor (ER) positive and negative tumors. During passaging of ER-positive tumors (four tumors), we observed that these tumors require estrogen supplements at the time of surgery to survive and grow. ER-negative tumors however, grew in the absence of added estrogen (data not shown; see also ref. 13).

In this study, human origin of tumor cells growing in the GFP was confirmed by an in situ hybridization analysis and using the anti-human keratin antibody, AE-3 (data not shown; the tumors analyzed are indicated in Table 1).

To determine if there were any factors useful in predicting whether a given patient's tumor would grow quickly, slowly, or not at all in SCID mice, we evaluated various available clinical data, including node status, ER expression, and whether or not the patients had previous treatment (e.g., chemotherapy) prior to surgical removal of the tumor.

We observed that of the 36 primary, untreated cancers transplanted (Table 1), 5 (14%) grew rapidly, 21 (58%) grew slowly, and 10 (29%) failed to grow. In comparison with six primary tumors from patients who had received chemotherapy previously (Table 1), four (67%) were rapid growers, two (33%) were slow growers, and none failed to grow ($P = 0.011$, Chi-square). Excluding metastatic lymph node xenografts, being ER-negative predicted rapid growth (8 of 19 [42%] ER-negative tumors grew fast while only 3 of 25 [12%] of ER-positive tumors did so; $P = 0.035$; Fisher exact test). The presence of axillary nodal metastasis was not predictive (5 of 28 node-positive tumors [18%] grew fast and 3 of 12 node-negative tumors [25%] did so; $P = 0.677$, Fisher exact test).

■ DISCUSSION

Experimental research on human breast cancer and the identification of better markers of tumor progression and risk assessment has been hampered by the lack of an animal model in which the growth of surgical specimens of primary recurrent breast carcinomas can be studied. Such an animal model could be used not only to study the cellular and molecular properties of various patients' breast carcinoma specimens, but to evaluate preclinically the effect of various chemotherapeutic agents, immunotherapies, or various environmental factors such as diet.

Most experimental models of breast cancer involve the use of rodent mammary tumors; these include models of spontaneous mammary tumor development, chemically and virally induced mammary carcinomas, and transgenic mice.[3] With regard to animal models of human breast cancer, the predominant model depends upon the use of a few well-studied cell lines that grow as solid tumors when inoculated into immunodeficient mice.[1-3] Although the breast tumor xenografts derived from cell lines are advantageous for many types of studies (primarily because the tumors are of human origin), relatively few human cell lines have been developed for

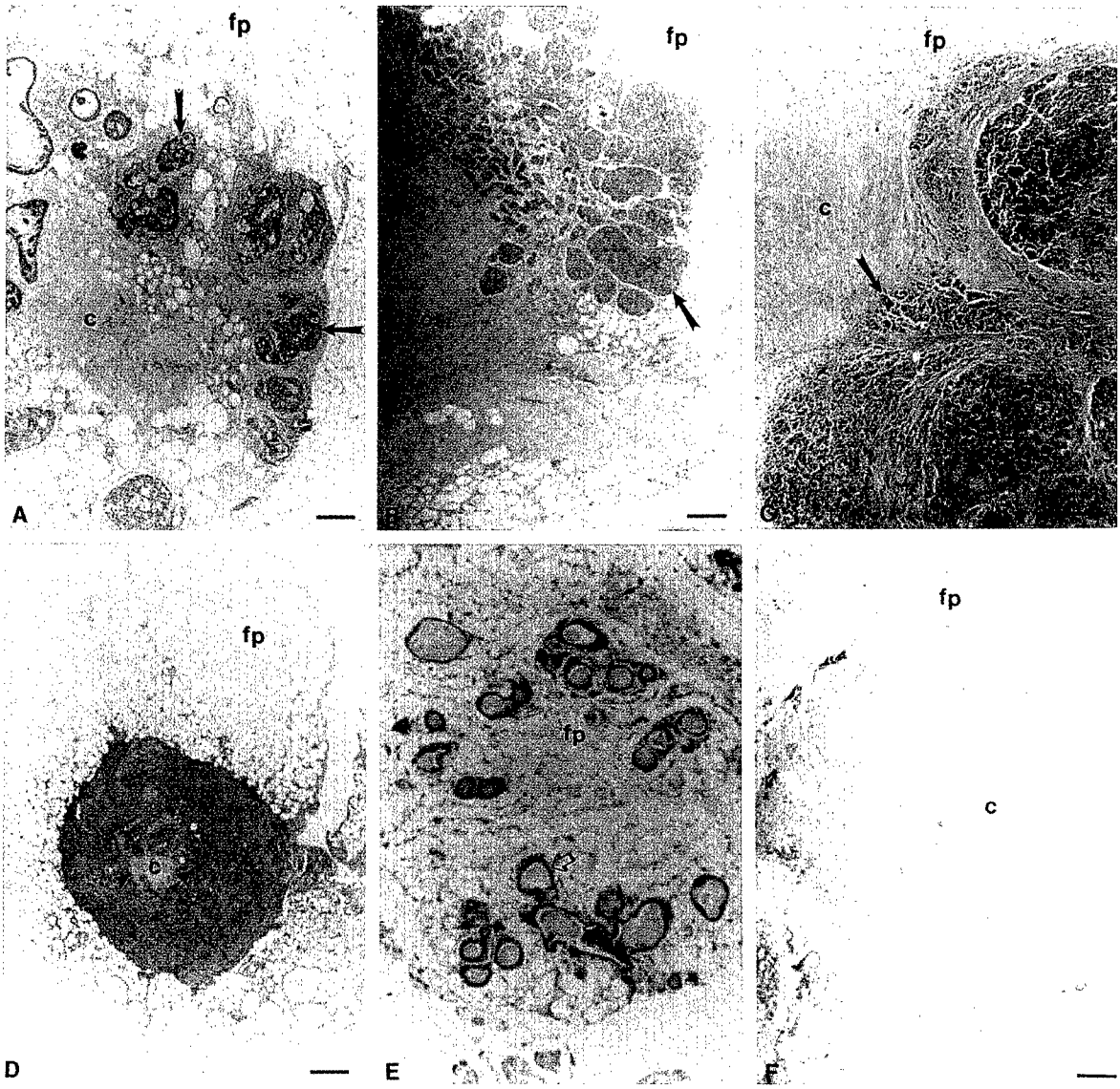

Figure 3 (A) Early pattern of tumor growth (7914) reveals numerous clusters of tumor cells at the periphery of the explant, but no tumor cells in the center of the collagen. (B, C) Representative slow-growth tumors exhibiting tumor growth from one region of the explant (7748 and 8180, resp). Again, there is little tumor remaining in the original explant. (D) Another example of slow-growth tumor (7869) in which tumor cells completely surround the region of the explant. (E) An example of apparently normal duct-like growth that occurs often in the various regions of the GFP implanted with breast tumor tissue (from 8180). (F) Representative histology of a tumor that did not grow after 3 months of engraftment. Only the original collagenous region of the implant remains, with little or no tumor cells visible. (c, collagen; fp, fat pad) (Bar = 60 μm in A, B, F; 40 μm in C; 75 μm in D)

this purpose, and most of these were derived from highly metastatic cells from effusions. It is clear from the abundance of studies using these human breast tumor cell lines that the factors that regulate the proliferation of these cells in vitro are significantly different from those that regulate normal human mammary epithelium,[2] and may also differ considerably from those that regulate tumor development in vivo. Thus, to acquire a thorough understanding of the biologic effects of the genetic alterations thought to be involved in breast cancer progression, it is essential to continue development and improvement of the existing experimental models, making it possible to isolate and study cells from various tumors on a more routine basis.

The histologic analyses presented in this paper indicate that placing intact pieces of surgical specimens of primary human breast carcinomas within the confines of the large SCID mouse GFP can often result in their sustained growth; for many tumors, the growth rate is fast enough to result in large amounts of tumor tissue that can be passaged into additional mice within weeks to months. For most of these tumors, metastatic spread within the mouse also occurred. The majority of tumors implanted exhibited a slower growth rate, with xenografts only doubling or tripling in size over a period of 6 months to 1 year or more.

The GFP was initially tested since it was a large, easily accessible fat pad that was histologically similar to

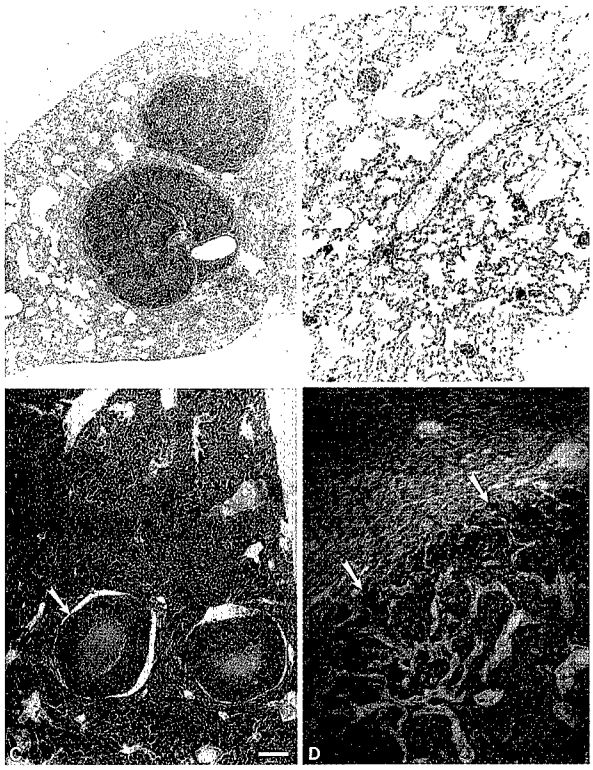

Figure 4 (A) Large foci of tumor growth are present within the lung parenchyma. This metastasis occurred during the 3rd passage of tumor specimen 7443. (B) Another example of lung metastasis: scattered small foci of tumor growth can be seen throughout the lung parenchyma. (C) Liver metastasis seen with tumor 7722. Note the similarity of metastatic tumor growth to that seen in this same tumor growing within the GFP (Fig. 1B). (D) Liver metastasis of tumor 8029 reveals a different histologic organization from that seen in Fig. C (arrows indicate regions of tumor growth seen in each case). (Bar = 40 μm)

the mammary fat pad, a site in rodents that has long been used by breast cancer biologists. However, unlike the mammary fat pad, the GFP is large enough to wrap completely about an intact segment of tumor, a factor we believe to be important for more successful tumor growth. Importantly, the GFP and the omentum are frequently the sites of metastasis of several types of cancers,[21] suggesting that this site may be rich in factors that sustain the growth of tumors. The large size and ease of accessibility of the GFP compared with the mammary fat pad would make this site preferable for various types of studies. A full comparison of both sites is warranted and is underway in our laboratory. The GFP of mice appears to be highly vascularized and consists largely of white fat cells (adipocytes) mixed with regions of brown fat and is similar in histology to the mammary fat pad, except that it is completely devoid of mammary epithelium; further, in SCID mice few other connective tissue cells were seen whereas in normal (Balb/c) mice, frequent macrophages and lymphoid cells were seen among the adipocytes throughout the tissue (E. Repasky, unpublished data). Many studies have been conducted on the metabolic importance of this organ in various rodents.[22,23] The interior of the gonadal fat pad may provide a particular microenvironment that stimulates or maintains breast tumor growth. Indeed, normal and malignant breast tissue develop near, and are maintained in close association with, fat cells in the mammary fat pad. Noteworthy in this regard is the fact that fat tissue can synthesize estrogen or harbor other factors that stimulate breast epithelium.[24-26] However, in this study we observed an estrogen-dependence of tumors that were ER-positive, whereas ER-negative tumors grew in the absence of estrogen supplementation. In the case of at least one ER negative tumor however, we have determined that it will grow more rapidly if estrogen is provided to the animal (R. Burd, Y. Xu, and E. Repasky, unpublished data).

The impaired immune status of the SCID mouse (lacking both T and B cells) mouse may also play an important role in enhancing tumor growth in the GFP, since preliminary data indicate that the SCID mouse GFP sustains growth better than a similar implantation in the nude mouse (T. Sakakibara and E. Repasky, unpublished data). Several reports indicate that various other tumors may grow better in the SCID mouse than in the nude mouse (see review in ref. 27). This may be due to the absence of both T and B cells within the SCID mouse, or to the lack of circulating xenoreactive antibodies that may interfere with breast tumor growth. SCID mice do however have an active natural immunity (including natural killer cells), and at this time, we do not know if the growth of breast xenografts can be enhanced by using NOD/LtSz-scid mice, in which the natural killer cell activity is significantly reduced.[28] However, preliminary data has shown increased growth potential in mice treated with anti-asialogmi, which blocks NK cell activity (R. Burd, S. Dziedzic, E. Repasky, unpublished data).

Occurrence of metastatic spread of tumors derived from some patients and not others may have important clinical implications; a longer term analysis of the clinical outcome of the patients used in this study will help to verify this hypothesis. Similarly, expression of various pathohistologic growth patterns of the xenografts as well as the differences in growth rate indicated by this study may directly reflect the different biologic properties and malignant potential of the original tumor specimens. That there is a heterogeneity of cell types within primary or locally recurrent tumors is exemplified by the fact that within the xenograft we sometimes found regions of cells that were similar in appearence to those found in normal mammary epithelium, sometimes in close proximity to obviously more invasive cells. We observed this normal growth pattern most often in association with the slow-growing tumors. Exactly how normal these cells are is open to question. But it is of interest to note that benign human breast tumors have been reported to grow in nude mice[13] and, in fact, were reported to grow more consistently than malignant specimens. It has also been noted that, although invasive cells from primary breast tumors are difficult to culture, cells

from benign lesions can be readily cultured.[29]

In this study, we analyzed several clinical factors that may be predictive of whether a tumor grows quickly or not at all. We found that a high proportion of tumors that grow quickly are ER-negative and derived from patients who had received previous chemotherapy. Since previous reports on the growth of patients' breast tumors in nude and SCID mice have not included this type of additional clinical data, we cannot compare our results or determine if these factors may play a role in the enhanced growth potential observed in this study. It is important to note that even with chemotherapy or the absence of estrogen receptors, tumors would still often fail to grow if implanted subcutaneously, so there appears to be a growth advantage for even these tumors if implanted within the gonadal fat pad. In one case, we derived primary untreated tumor and primary tumor following chemotherapy from the same patient (compare tumor 7595 with 7744). Although this patient's untreated primary tumor failed to grow in 3 mice, her chemotherapy-treated primary tumor exhibited slow growth in 6 out of 10 mice. These results suggest that future reports should include additional clinical data with rgard to patient specimens that are used, since these factors may play a role in whether a tumor grows quickly, slowly, or not at all.

Numerous questions are raised by this study, such as the possible role of vascularization in regulating whether a tumor grows quickly, slowly, or not at all, or what the relationship is (if any) exists between prior chemotherapy, estrogen-receptor status, and growth rate of the xenograft. We are currently conducting an immunocytochemistry analysis of cell surface markers of clinical interest, such as HER-2/neu and p-glycoprotein expression, among the various xenografts using paraffin-embedded material that has been prepared from each. These and other data may be useful in learning more about the factors that regulate the growth rate and metastatic potential of the various xenografts. However, we believe that the use of the SCID mouse gonadal fat pad model, as described here, can provide an easily accessible site for growing and passaging many human breast carcinomas. The ability to collect large amounts of tumor tissue from various passages and from metastatic sites in this model will be useful for genetic, biochemical, and immunologic analyses of breast tumor cells; moreover, the ability to subpassage human breast tumors into large numbers of mice will make possible a variety of experimental studies to evaluate new therapeutic approaches for this disease.

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